# HPLC Separation and Spectral Characterization of Browning Pigments from White Grapefruit Juice Stored in Glass and Cans†

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A ternary solvent system consisting of water, acetonitrile, and tetrahydrofuran was used in HPLC to separate browning pigments formed in grapefruit juice stored in cans and bottles. Pigments were spectrophotometrically characterized using a photodiode array detector. HPLC runs evaluated at various storage times manifested different browning patterns with both the canned and bottled juices. Some compounds increased and then decayed with time, whereas others continued to intensify. Late eluting components (Rt > 80 min) were the most nonpolar browning pigments and, also, showed the most consistent HPLC patterns. HPLC patterns, retention times, and UV–vis spectra of browning components in canned juices were notably different from those of glass-packed juices. Most of the separated compounds exhibited absorbance maxima in the region 380–400 nm, and a value of 390 nm was ascertained by averaging all visible-absorbing maximums. No browning pigment had a distinct absorbance above 400 nm. The use of 390 nm to monitor nonenzymic browning of grapefruit juice is more appropriate than the current accepted value of 420 nm.

## INTRODUCTION

Under nonrefrigerated storage conditions, processed grapefruit juice darkens with increasing storage time due to the formation of browning pigments. This nonenzymic browning has been a visual defect of processed grapefruit juice and is one of the factors that determines shelf life. Browning in grapefruit juice is also accompanied by an increase in off-flavors, which also limits shelf life (Nagy et al., 1989).

Browning in citrus juices has been studied by a number of investigators (Joslyn, 1957; Wolfrom et al., 1974; Saguy et al., 1978; Lee and Nagy, 1988; Nagy et al., 1989) and the subject has been reviewed recently by Handwerk and Coleman (1988). There is considerable disagreement as to which reaction predominates in nonenzymic browning, i.e., the sugar–amino acid reaction of the Maillard type or ascorbic acid degradation. The vast majority of citrus browning studies have attempted to investigate the initial reactants which are associated with, but do not immediately produce, browning pigments. By adding specific components to juice or model systems, the compounds that accelerate or inhibit browning have been identified. Although many studies have been conducted on general procedures to assess nonenzymic browning of citrus juices, few studies have defined the physicochemical nature of the individual browning pigments. Rouseff and co-workers (Rouseff et al., 1989) were the first investigators to develop an HPLC procedure to resolve and define the polar nature of browning pigments in heat-abused grapefruit juice. However, the physicochemical characteristics of those browning pigments were not addressed during that study. In addition, while it is generally known that browning is more of a problem in glass containers than in tin-plated steel cans, the number or types of browning pigments formed in each case are unknown.

The purpose of this study was to develop a procedure that would examine time-related HPLC patterns and UV–vis properties of browning pigments formed in temperature-aged grapefruit juice. A secondary goal included an examination of the UV–vis properties of container-specific pigments.

## EXPERIMENTAL PROCEDURES

**Samples.** Cans (6 fl oz) and bottles (7 fl oz) of commercial white grapefruit juice were obtained from a Florida citrus processor. Both canned (tin-plated with enamel-coated lids) and bottled juices were reconstituted from separate concentrate lots prepared 1 week apart. All glass bottles were stored in the dark in temperature-controlled cabinets. Light was not a variable contributing to differences between cans and bottles. Canned grapefruit juice samples stored for 21 weeks at 4 and 40 °C were used to obtain the visible absorption profile (Figure 1) of total nonenzymic browning pigments. For HPLC and UV–vis spectra studies (Figures 2–5), cans were stored for up to 12 weeks at 60 °C, whereas the glass bottles were stored for up to 15 weeks at 60 °C. Control samples were stored at 4 °C. Storage temperatures of 40 and 60 °C were selected on the basis of previous studies (Rouseff et al., 1989; Nagy et al., 1990) of accelerated brown pigment formation. For each storage time, random duplicate samples were analyzed. Canned juices were analyzed every week, whereas bottled juices were analyzed every 3 weeks.

**Sample Preparation for HPLC.** A 35-mL sample of canned grapefruit juice was centrifuged for 120 min at 3170*g*. The supernatant fluid was decanted into a 1-L round-bottom flask and freeze-dried according to the method of Klim and Nagy (1988). The dried sample was then slurried with 35 mL of methyl alcohol; all material did not dissolve. The slurry was filtered through Whatman No. 42 filter paper. Then 4.0 mL of the filtrate was evaporated under nitrogen at 25 °C to a volume of 1.0 mL and filtered through a 0.45-mm filter. The filtrate was ready for HPLC analysis.

**Reagents and Chemicals.** All solvents were of HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ). Laboratory-deionized water was further purified using a Milli-Q (Millipore Corp., Milford, MA).

**Equipment.** Two high-pressure pumps (Models 6000A and 510), a system controller (Model 721), and an automated sample injector (Wisp, Model 710B) (Waters Associates, Milford, MA) were used. A Hewlett-Packard Model 1040A HPLC photodiode array detector system with a plotter (HP 7470) and flexible disk drive (HP 89201M) (Hewlett-Packard, Palo Alto, CA) were used

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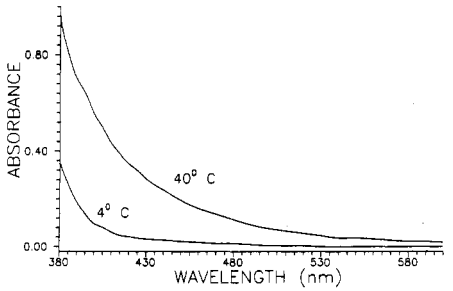


**Figure 1.** Visible absorption spectra of control grapefruit juice stored at 4 and 40 °C.

for real-time spectral data acquisition. An ultrasonic cleaner (Cole-Parmer, Chicago, IL, Model 8845-6) and international clinical centrifuge (Model CL) were used.

**Chromatographic Conditions.** A 3-μm Supelco (Bellefonte, PA) $C_{18}$ column, 4.6 mm i.d. × 150 mm, was used with a Brownlee (Santa Clara, CA) Spheri-5 $C_{18}$ precolumn, 4.6 mm i.d. × 50 mm. A Supelco low dead volume in-line filter was used in front of the precolumn to trap particulates. A ternary solvent system consisting of water, acetonitrile, and a small amount of tetrahydrofuran (THF) was used to develop the solvent gradient. The specific gradient program used is shown in the following outline:

| time, min | % 99.5% water:0.5% THF (v/v) | % acetonitrile |
|---|---|---|
| 0 | 100 | 0 |
| 5 | 99 | 1 |
| 38 | 91.6 | 8.4 |
| 78 | 78.1 | 21.9 |
| 98 | 67.9 | 32.9 |
| 103 | 3.3 | 96.7 |
| 106 | 0 | 100 |

Flow rate was 1.0 mL/min. All gradient changes were accomplished linearly. The column was equilibrated for at least 15 min before the next sample was injected. Sample injection volumes were 10 or 20 μL depending upon concentration. The eluant was monitored at 398 nm with an attenuation of 0.005 AU (absorption unit). Peak width was 0.3 min, and the threshold was 10 mAU (milli absorption unit). Solvents were degassed using an aspirator vacuum in an ultrasonic bath.

**Absorption Spectra of Total Browning Pigments.** Absorption spectra of control canned grapefruit juice (4 °C; 21 weeks of storage) and of heat-treated canned grapefruit juice (40 °C; 21 weeks of storage) were derived from freeze-dried samples according to the method of Klim and Nagy (1988). Samples, dissolved in methanol, were read between 380 and 600 nm with a Bausch and Lomb Spectronic 88 using 13-mm cuvettes (10 mm light path).

## RESULTS AND DICUSSION

The processed white grapefruit juices used in these studies possessed the following properties: canned, 10.3 °Brix, 1.12% citric acid, pH 3.36, Brix/acid ratio 9.2; bottled, 9.9 °Brix, 0.99% citric acid, pH 3.48, Brix/acid ratio 10. The juice of white-fleshed grapefruit contains virtually no colored pigments (Gross, 1977, 1987; Romjaro et al., 1979), and therefore, pigment interferences within the visible-wavelength region are minimal (Klim and Nagy, 1988). The absorption spectra of a 4 °C stored (control) and 40 °C (heated) grapefruit juice are noted in Figure 1. From the absorbance vs wavelength profile, it is evident that there is some absorbance of components in the control sample, i.e., from a high absorbance (Abs) of 0.36 Abs (380 nm) to a low of 0.03 Abs (≥440 nm). In contrast, the absorbance of the heat-treated sample is highly intensified; values range from 0.96 Abs (380 nm) to 0.03 Abs (560 nm). Interestingly, there does not appear to be a distinct visible-absorbing wavelength maximum for this mixture of browning pigments. The absorbance vs wavelength profile has an asymptotic decline and, according to Meydav et al. (1977), is not affected by the different methods of extraction and clarification of the browning pigments.

**Bottled Grapefruit Juice.** Figure 2 illustrates the trend in formation of browning pigments with storage time. Samples were stored at 60 °C and monitored at 0, 3, 6, 9, 12, and 15 weeks. The control sample (zero-week storage) manifested very few peaks when monitored at an attenuation of 5 mAU. Peaks occurring at the beginning of the chromatographic run (0–3 min) represent highly polar compounds but with limited absorption in the visible region and no noticeable brown coloration. Two defined peaks were noted at retention times of 53 and 58 min. On the basis of the UV–vis scan of peaks and the lack of any noticeable absorption in the visible region, the control sample appeared to be devoid of browning pigments.

After 3 weeks of storage, about 30 new peaks were discernible. The number of peaks progressively increased as storage time increased, and after 15 weeks of storage, well over 100 peaks were detected. The gradient mobile phase employed in our study elutes polar components first and the most nonpolar, polymeric browning pigments last (Rouseff et al., 1989). Compounds eluting between 0 and about 50 min are considered to be polar or semipolar in nature. No pattern relating formation or decay of specific components in this region was noted. Peaks eluting in the region from about 50 to 106 min increased in number with storage time. Peak heights for some components increased regularly, whereas others decreased. Peaks in the region between 82 and 93 min appeared more discrete and showed progressive increases in intensity with time.

Figure 2 imparts the impression that some compounds increase and then decay with time, whereas others continue to intensify with time. Several compounds are transient intermediates, i.e., compounds that rapidly become consumed by degradative and/or polymerization reactions under the conditions of high temperature. The progressive increase in the intensities of peaks eluting after about 86 min indicates increased formation of the most nonpolar browning pigments. This observation was previously noted by Rouseff et al. (1989).

Figure 3 represents UV–vis spectra of 16 distinct chromatographic peaks. The compounds (Ct, A–O) are the same compounds labeled in Figure 2. Components were resolved by HPLC and evaluated by photodiode array detection. All peaks were carefully selected, and no duplication occurred (ascertained by retention time and by the UV–vis spectrum). Reproducibility of peak retention times in the region 60–105 min showed a 0.6–1.2% range for the coefficients of variation.

Table I summarizes the characteristics of the browning pigments. At 0 weeks of storage, the selected "control" peak manifested a distinct UV spectrum with absorption maxima at 228, 266, and 334 nm and no distinct visible-absorbing wavelength. Only minor absorption in the visible-wavelength region (>380 nm) was noted. At 3 weeks of storage, peaks B and C with absorptions in the visible region became manifest. However, highly polar peak A eluting at 6.9 min showed no visible-absorbing wavelength. A scan of other peaks in the retention region 0–10 min also confirmed that early eluting, highly polar components generally possess negligible visible-absorbing characteristics. At 6 weeks, three selected spectra (peaks D–F—based on size and chromatographic placement) showed some visible absorption, although peak E possessed a weak, broad, nondescript absorption band that tailed into the visible region.

Peaks H and I monitored at 9 weeks of storage showed major visible absorptions, whereas peak G showed no distinct visible absorption band. As a tentative rule, peaks with retention times greater than 80 min generally showed

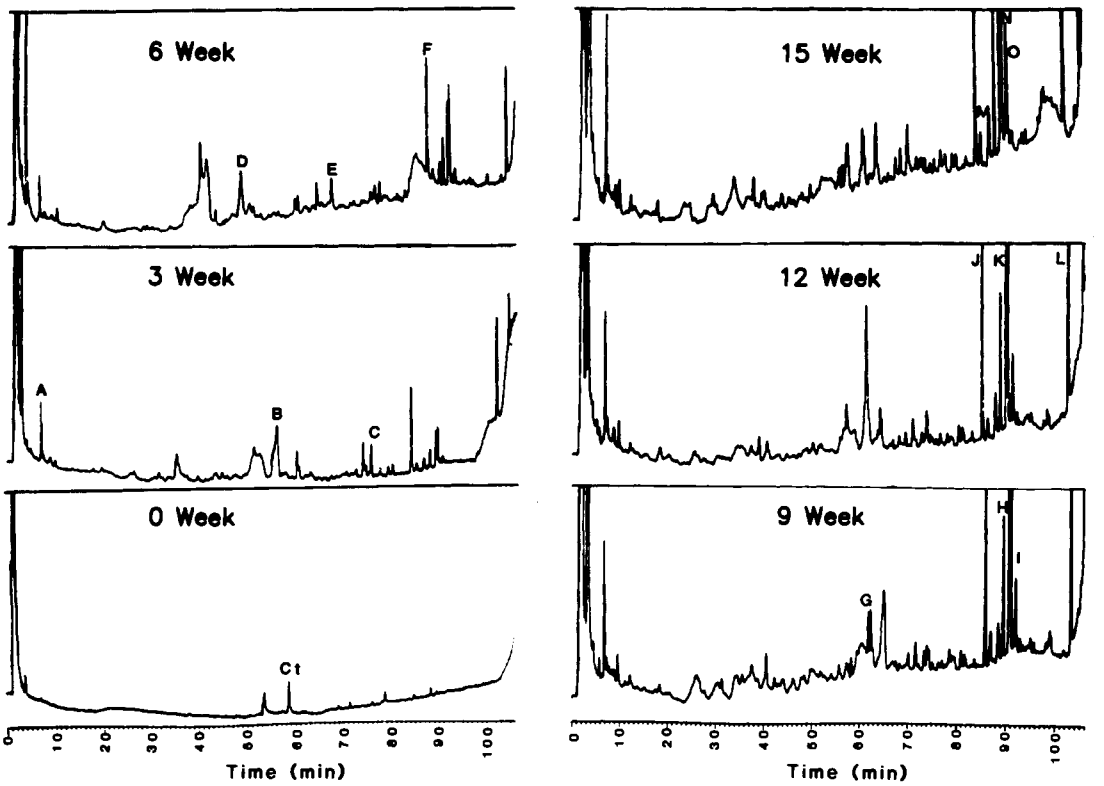


**Figure 2.** Formation of browning pigments in bottled grapefruit juice stored at 60 °C over a 15-week storage period. Peaks for analysis were randomly selected from 1 control (Ct) and 15 browning compounds (A–O). Each selected peak was specific and was not replicated. Browning compounds analyzed by photodiode array detection include 0 week (Ct), 3 week (A–C), 6 week (D–F), 9 week (G–I), 12 week (J–L), and 15 week (M–O).

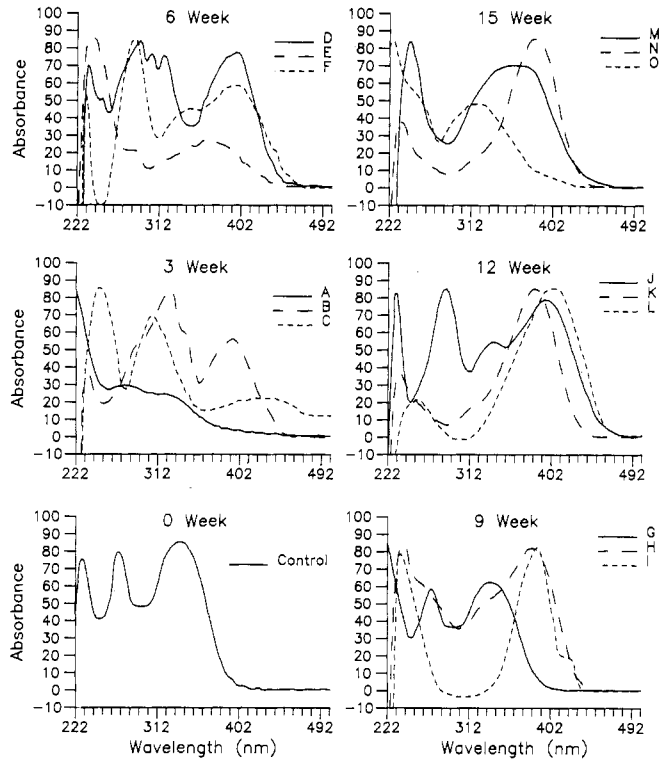


**Figure 3.** UV–vis spectra of 16 compounds separated by HPLC from bottled grapefruit juice and evaluated by photodiode array detection. The compounds (Ct, A–O) are the same labeled compounds noted in Figure 2.

some form of visible absorption. The buildup of these peaks in this retention region is reflected by Figure 2. Peaks J–L at 12 weeks and peak N at 15 weeks of storage have distinct visible absorption bands, whereas peaks M and O are less distinct. It is evident after monitoring of many browning components from heat-abused, bottled

**Table I. Characteristics of Browning Pigments in Bottled Grapefruit Juice**

| | | | abs max, nm | |
|---|---|---|---|---|
| week | peak ID | Rt, min | UV | vis |
| 0 | control | 58.6 | 228, 266, 334 | none |
| 3 | A | 6.9 | 222 | none |
| 3 | B | 55.6 | 232, 326 | 392 |
| 3 | C | 75.3 | 246, 304 | 432 (broad) |
| 6 | D | 48.7 | 234, 290, 303, 318 | 400 |
| 6 | E | 67.3 | 234, 370 | none distinct |
| 6 | F | 87.2 | 230, 282 | 402 (broad) |
| 9 | G | 62.4 | 222, 268, 334 | none |
| 9 | H | 89.5 | 238 | 382 |
| 9 | I | 91.6 | 234 | 388 |
| 12 | J | 85.1 | 228, 282, 334 | 400 |
| 12 | K | 90.3 | 234 | 382 |
| 12 | L | 102.6 | 252 | 398 |
| 15 | M | 84.9 | 246, 362 | 382–402 (broad) |
| 15 | N | 89.4 | 232 | 382 |
| 15 | O | 90.5 | 226, 318 | none |

grapefruit juice that visible absorption maxima are confined to the region from 380 to about 400 nm.

**Canned Grapefruit Juice.** Figure 4 illustrates formation of browning pigments with storage time. Samples were stored at 60 °C and monitored weekly for 12 weeks, but only samples monitored at 0, 3, 5, 7, 9, and 11 weeks are presented in Figure 4. The canned control sample (0 weeks of storage) was similar in its HPLC pattern to the bottled control sample (Figure 2) but with higher peak intensities. The largest peak in the canned control sample is located at 58.6 min and is identical (by retention and UV–vis spectrum) to the control peak of the bottled sample (Figure 3; Table I). UV–vis scans of 10 distinct peaks (Rts = 6.9, 7.4, 52.7, 53.6, 58.6, 68.9, 71.3, 78.7, 84.7, and 88.2 min) confirmed no discrete absorbing bands in the

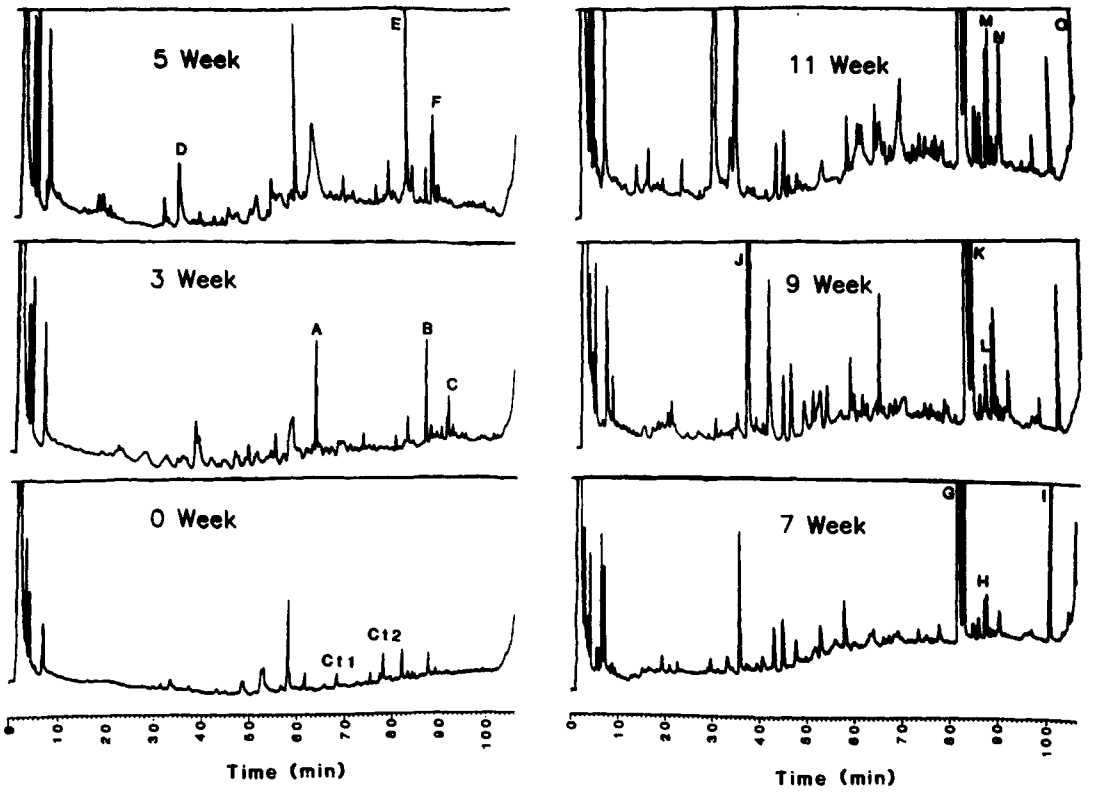


**Figure 4.** Formation of browning pigments in canned grapefruit juice stored at 60 °C over an 11-week storage period. Browning compounds analyzed by photodiode array detection include 0 week (Ct 1, Ct 2), 3 week (A–C), 5 week (D–F), 7 week (G–I), 9 week (J–L), and 11 week (M–O).

visible region. Therefore, it was concluded that the canned control sample lacked browning pigments.

As storage time lengthened, varying chromatographic patterns emerged. Peaks eluting between 10 and about 70 min showed no consistent pattern. This is evidenced by chromatographic runs for 3, 5, 7, 9, and 11 weeks. The two prominent peaks (Rt = 63.6 and 86.8 min) for 3 weeks were barely discernible in the 5-week samples. The most defined pattern to emerge occurred with the 7-, 9-, and 11-week samples in the retention area between 80 and 105 min. This area (80–105 min) was also prominent with glass-packed grapefruit juice (Figure 2). However, the pattern, retention times, and UV–vis spectra of components in these canned juices (Figure 4) were notably different from those of glass-packed juices (Figure 2).

The formative and degradative scenarios of pigments in canned juices were similar to those of glass-packed juices. In the elution region between 10 and about 70 min, compounds form and decay at different rates. These compounds may be considered intermediates of complex polymerization reactions leading to browning pigments. Nagy and co-workers (Nagy et al., 1990a) postulated that the browning mechanisms in citrus juices involve a complex group of reactants and intermediates that yield an assortment of brown pigments of highly unstable characteristics. Rouseff et al. (1989) observed that the late eluting, nonpolar pigments (Rt > 100 min) appeared to be major contributors to the browning observed in canned grapefruit juices.

Figure 5 illustrates UV–vis spectra of 17 distinct chromatographic peaks analyzed over the 0–11-week storage period. The compounds (Ct 1, Ct 2, A–O) are the same compounds labeled in Figure 4. The characteristics of these compounds are summarized in Table II. For 0 weeks of storage, two peaks were selected as controls, namely, control 1 (Rt = 68.9 min) and control 2 (Rt = 78.7 min). Neither control, while exhibiting distinct UV-absorbing bands, possessed any discrete visible-absorbing band.

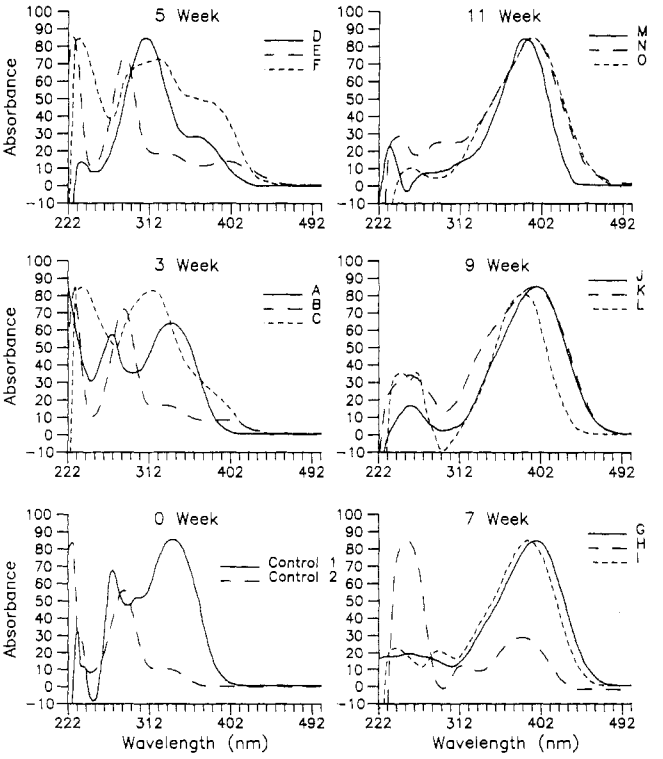


**Figure 5.** UV–vis spectra of 17 compounds separated by HPLC from canned grapefruit juice and evaluated by photodiode array detection. The compounds (Ct 1, Ct 2, A–O) are the same labeled compounds noted in Figure 4.

Random selection of three peaks from the 3-week-stored sample is noted in Figure 5. Peaks A, B, and C (63.3, 86.8, and 91.7 min, respectively), while showing distinct UV-absorbing bands, did not exhibit any noticeable absorption

**Table II. Characteristics of Browning Pigments in Canned Grapefruit Juice**

| week | peak ID | Rt, min | abs max, nm UV | abs max, nm vis |
|---|---|---|---|---|
| 0 | control 1 | 68.9 | 233, 265, 335 | none |
| 0 | control 2 | 78.7 | 226, 282 | none |
| 3 | A | 63.3 | 222, 268, 336 | none |
| 3 | B | 86.8 | 230, 292 | none |
| 3 | C | 91.7 | 234, 322 | none |
| 5 | D | 34.0 | 233, 306, 372 | none |
| 5 | E | 82.5 | 226, 284 | none |
| 5 | F | 88.2 | 234, 322 | 385 (broad) |
| 7 | G | 83.5 | none distinct | 394 |
| 7 | H | 87.4 | 254, 314 | 384 |
| 7 | I | 102.0 | none | 392 |
| 9 | J | 36.9 | 258 | 394 |
| 9 | K | 84.0 | 260 | 394 |
| 9 | L | 86.8 | 242, 266 | 382 |
| 11 | M | 87.6 | 234 | 382 |
| 11 | N | 90.6 | 242 | 386 |
| 11 | O | 105.6 | 254 | 390 |

in the visible region. In contrast, the 3-week stored, glass-packed juice (Figure 3) manifested distinct pigments with visible-absorbing bands. A possible explanation might be due to the packaging container. Previous research by Nagy et al. (1990b) showed that during the early stages of storage of grapefruit juice in tin-plated cans the tin metal minimized brown pigment formation. This favorable property of tin was due to its oxidation–reduction potential and to its ionic-binding ability. Peaks D–F of the 5-week sample did not show distinct visible-absorbing bands, although peak F manifested significant absorption between 390 and 410 nm.

Samples monitored at later storage periods showed components with discrete visible-absorbing properties. At the 7 weeks, peaks randomly selected at 83.5, 87.4, and 102.0 min all had visible absorption bands in the region between 384 and 394 nm. Random selection of peaks for the 9 (peaks J–L) and 11 weeks (peaks M–O) all showed defined visible absorption maxima with a range between 382 and 394 nm.

**Bottled vs Canned Browning Pigments.** Examination of HPLC profiles of browning pigment patterns in bottled vs canned juice attests to considerable differences. In bottled juices, minimum formation of components is noted in the elution region between 30 and 50 min (Figure 2) at 9–12-weeks of storage. However, with canned juices (Figure 4) noticeable components elute in this region during storage at 7–11 weeks. With bottled juices, major nonpolar components are noted in the elution region 85–93 min, whereas, with canned juices, these major nonpolar components elute between 80 and 87 min. The retention time differences appear to be small but are consistent and reproducible. Although we did not compare every browning pigment in bottled juice to every browning pigment in canned juice, several pigments (randomly selected) in the region 80–90 min were compared (Figures 3 and 5) and found to exhibit different UV–vis spectra. We concede, however, the possibility that some pigments may be common to both canned and glass-packed juices.

**Browning Pigments in Grapefruit Juice.** It is difficult to assemble coherent mechanisms to define brown pigment formation in grapefruit juice. The complexity of components is attested by the HPLC profiles of bottled (Figure 2) and canned (Figure 4) grapefruit juices. Formation, as well as degradation, of numerous precursors, intermediates, and transient browning compounds occurs during nonenzymic browning. Examination of 33 chromatographic peaks (Figures 3 and 5) by UV–vis spectroscopy yields a bewildering array of spectra. Surprisingly, very little is known about the chromophores present in these browning pigments. Unsaturation and polarizibility are essential features of a chromophore exhibiting color. Although auxochromes enhance or deepen color, high-intensity absorption within the visible-wavelength region is generally associated with the conjugated systems of the chromophores. A molecule absorbing radiation in the visible region generally requires the conjugation of three or more simple chromophores, although there are limited exceptions to this rule (Gillam and Stern, 1958). Examination of the UV–vis spectra in Figures 3 and 5 indicates that the chromophoric groups of the visible-absorbing compounds might possess similar structural properties because of the relative narrow range of the visible absorption band region (382–400 nm).

Examination of compounds with noticeable visible-absorbing bands (Tables I and II) indicates that a majority absorb in the region between 382 and 400 nm. To this end, a value of 390 nm was ascertained by averaging visible-absorbing maxima from Tables I and II. The use of 390 nm to monitor nonenzymic browning of grapefruit juice is more appropriate than the standard value of 420 nm. Therefore, we suggest substituting 390 nm for 420 nm in nonenzymic browning determinations.

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Received for review July 22, 1991. Accepted October 28, 1991.